

Impact of DHA intake in a mouse model of synucleinopathy

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ABSTRACT

Polyunsaturated fatty acids omega-3 (n-3 PUFA), such as docosahexaenoic acid (DHA), have been shown to prevent, and partially reverse, neurotoxin-induced nigrostriatal denervation in animal models of Parkinson's disease (PD). However, the accumulation of α -synuclein (α Syn) in cerebral tissues is equally important to the pathophysiology. To determine whether DHA intake improves various aspects related to synucleinopathy, ninety male mice overexpressing human α Syn under the Thy-1 promoter (Thy1- α Syn) were fed one of three diets (specially formulated control, low n-3 PUFA or high DHA) and compared to non-transgenic C57/BL6 littermate mice exposed to a control diet. Thy1- α Syn mice displayed impaired motor skills, lower dopaminergic neuronal counts within the substantia nigra (-13%) in parallel to decreased levels of the striatal dopamine transporter (DAT) (-24%), as well as reduced synaptic proteins PSD-95 (-51%) and synaptophysin (-80%) in the cerebral cortex compared to C57/BL6 mice. However, no significant difference in dopamine concentrations was observed by HPLC analysis between Thy1- α Syn and non-transgenic C57BL/6 littermates under the control diet. The most striking finding was a favorable effect of DHA on the survival/longevity of Thy1- α Syn mice (+51% survival at 12 months of age). However, dietary DHA supplementation did not have a significant effect on other parameters examined in this study, despite increased striatal dopamine concentrations. While human α Syn monomers and oligomers were detected in the cortex of Thy1- α Syn mice, the effects of the diets were limited to a small increase of 42 kDa oligomers in insoluble protein fractions upon n-3 PUFA deprivation. Overall, our data indicate that a diet rich in n-3 PUFA has a beneficial effect on the longevity of a murine model of α -synucleinopathy without a major impact on the dopamine system and motor impairments, nor α Syn levels.

Keywords: polyunsaturated fatty acids omega-3, α -synuclein, Parkinson, docosahexaenoic acid, Thy1- α Syn, synaptic proteins, dopamine

1. INTRODUCTION

A number of epidemiological and preclinical studies suggest that consumption of diets enriched in n-3 polyunsaturated fatty acids (PUFA) may decrease the risk of developing neurodegenerative diseases such as Parkinson's disease (PD) (Bousquet et al., 2011a; Bousquet et al., 2011b; Calon and Cicchetti, 2009; Coulombe et al., 2016; de Lau et al., 2005; Dyall, 2015). Most evidence of neuroprotection by n-3 PUFA has been collected in animal models of PD supplemented with docosahexaenoic acid (DHA; 22:6 n-3, or cervonic acid) and/or eicosapentaenoic acid (EPA, 20:5 n-3) (Bousquet et al., 2011a; Bousquet et al., 2008; Dyall, 2015; Seidl et al., 2014). Our group has previously shown that a formulation containing DHA and EPA (4:1 ratio) prevents nigral toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP) - a compound classically used to reproduce dopaminergic-associated features of PD in mice (Bousquet et al., 2011a; Bousquet et al., 2009; Bousquet et al., 2011b; Bousquet et al., 2008). This is supported by additional reports in the MPTP-probenecid model exposed to EPA (Luchtman et al., 2012). More recently, we have shown that DHA induces a partial recovery of the dopaminergic system following a 6-hydroxydopamine lesion (Coulombe et al., 2016), suggesting that DHA not only has a neuroprotective effect but can also act as a neurorestorative compound. The mechanisms underlying the beneficial properties of DHA include neurotrophic factor secretion (Bousquet et al., 2009), dampening of the inflammatory cascades (Calder, 2013), prevention of oxidative damage (Cardoso et al., 2014), and stimulation of cell survival pathways (Akbar et al., 2005), all processes potentially involved in the cell death characteristic of PD. Beside possible effects on mood-related symptoms (da Silva et al., 2008), no clinical intervention trial has confirmed a neuroprotective effect of n-3 PUFA in PD.

In addition to the prominent dopaminergic cell loss observed in PD, pathological hallmarks of the disease include abnormal levels and/or phosphorylation of the protein α -synuclein (α Syn), which

is now the target of various therapeutic developments (Dehay et al., 2015; Spillantini and Goedert, 2016; Vaikath et al., 2015). Indeed, genetic data suggest that accumulation of α Syn deposits may play a causal role in PD. For example, increased α Syn levels generated by gene duplication or triplication lead to familial forms of PD (Lee and Trojanowski, 2006; Ross et al., 2008; Singleton et al., 2004). Cellular α Syn levels accumulate with aging prior to the formation of inclusions, suggesting that blocking age-related accumulation of non-aggregated α Syn may alter the progression of PD (Chu and Kordower, 2007). In sporadic forms of PD, α Syn accumulation and deposition into Lewy bodies is a neuropathological hallmark of the disease, essential for its definitive diagnosis (Dickson et al., 2009). Equally important, α Syn has been shown to trigger neuroinflammation, as well as mitochondrial dysfunction and oxidative stress, which in turn may contribute to neuronal degeneration (Dehay et al., 2015). Conversely, there is a significant amount of literature supporting the hypothesis that n-3 PUFA corrects neuroinflammation and oxidative stress (Bousquet et al., 2011a; Calon et al., 2004; Delattre et al., 2010; Lalancette-Hébert et al., 2011; Mori et al., 2017; Trepanier et al., 2016). Furthermore, a meta-analysis has established that intake of n-3 PUFA is associated with a lower PD risk (Kamel et al., 2014). However, previous reports also suggest that DHA enhances α Syn oligomerization (Yakunin et al., 2012). These studies combined provide compelling arguments that DHA or oxidized metabolites interact specifically with α Syn, thereby influencing its 3D conformation, its oxidant scavenger activity and possibly its propensity to form aggregates and Lewy bodies (Assayag et al., 2007; De Franceschi et al., 2017; 2009; Ruiperez et al., 2010; Sharon et al., 2003). Therefore, it is critical to determine whether dietary PUFA improve or exacerbate cerebral synucleinopathies *in vivo*.

While the MPTP mouse model of PD generates sufficient dopaminergic cell loss to test the neuroprotective capacities of various compounds, it fails to mimic other aspects of the disease

such as the accumulation of α Syn (Bezard and Przedborski, 2011; Bezard et al., 2013). To palliate to this important shortcoming of toxin-induced models of PD, different lines of mice overexpressing α Syn have been generated (Chesselet et al., 2012; Masliah, 2000). Among these transgenic germlines, the mouse overexpressing wild-type human α Syn under the neuron-specific Thy1 promoter (Thy1- α Syn) has been extensively characterized (Chesselet et al., 2012; Masliah, 2000) and has been shown to display high levels of α Syn and proteinase K-resistant inclusions containing α Syn in several brain regions, including the substantia nigra (Chesselet et al., 2012; Masliah, 2000). Although frank nigrostriatal cell loss has not been reported (Chesselet et al., 2012), Thy1- α Syn mice exhibit robust behavioral deficits under challenging motor tests, such as the beam traversal and pole tests, as well as signs of inflammation, mitochondrial dysfunction, and a broad range of deficits in non-motor domains affected by PD (Chesselet et al., 2012; Fleming et al., 2004). Thus, the Thy1- α Syn model stands as one of the best animal models to study α -synucleinopathies associated with PD.

We therefore sought to evaluate the effects of a nutraceutical intervention with DHA in the Thy1- α Syn mouse to determine whether the diet has a 'disease-modifying' effect on synucleinopathy, motor deficits and the dopaminergic nigrostriatal system.

2. MATERIALS AND METHODS

2.1 Animals

Male Thy1- α Syn mice (n=90) and non-transgenic (C57BL/6 NonTg, n=31) littermates were bred in our animal research facility from Thy1.2- α Syn mice (line 61) - obtained from Dr E. Masliah (UCSD) (Hashimoto et al., 2003) - on a full C57BL/6 background. Since the transgene is located on chromosome X, only males were selected for experimentations, while females were used for breeding. Additional male C57BL/6 were ordered from Jackson Laboratories as breeders. Mice

were housed in ventilated cages (between two to five animals per cage), on a 12:12h dark:light cycle in a vivarium dedicated to mice. All animals had free access to water and fed with their respective diets (formulated control, no DHA or enriched in DHA, see details in sections below). All experiments were performed in accordance with the Canadian Guide for the Care and Use of Laboratory animals and all procedures were approved by the animal research committee of the Centre de recherche du CHU de Québec.

2.2 Diets

Thy1- α Syn mice were either fed with a control (n=31), low n-3 PUFA (n=30) or a DHA-enriched diet (n=30) for ten months, beginning at 2 months of age until their sacrifice at twelve months. They were compared with C57BL/6 non-transgenic littermates fed the control diet (n=31). DHA was obtained in a microencapsulated formulation (1:25 ratio of EPA to DHA) to avoid oxidation (DSM Nutritional Products) and the pelleted chow was generated by Research Diets Inc. (NJ, USA). We used only purified diet formulations that were standardized to ensure consistency and eliminate batch-to-batch variation. Both diets were isocaloric and contained similar concentrations of macronutrients, vitamins and minerals. The dose of DHA was approximately ~0.8 g/kg/daily per animal. Detailed descriptions of diet contents are provided in **Table 1**.

2.3 Behavioral measures

Behavioral testing began when mice reached four months of age - two months after the animals were put on their specific diets - and was repeated at eight and twelve months. For the behavioral measures, the number of animals used for each testing session may differ due to exclusion based on each test criteria, death of the animal or unavailability of the experimenters at preset timepoints. All tests were performed in the morning and on consecutive days (a single test per day), by experimenters blind to treatment conditions. The animals were evaluated for

spontaneous activity where they were placed in a cylinder of ten centimeters in diameter for a 3-minute observational period. The number of contacts made by each paw against the glass walls were counted, as is done with unilateral 6-hydroxydopamine lesioned animals, to investigate limb movement asymmetry due to dopamine impairments (Glajch et al., 2012).

The beam test was adapted from previous beam-walking tests (Drucker-Colín and García-Hernández, 1991). The beam was built from Plexiglas and consisted of four sections of 25 cm each for a total length of 1 m. Each section had a different width, starting from 3.5 cm and decreasing by 0.5 cm in each section, reaching a width of 1.5 cm on the last section of the beam. Animals were trained to cross the entire length of the beam for 2 days at a frequency of 5 trials per day. On the third day, the test was performed and filmed. The videotapes were subsequently analyzed for the number of steps made by each animal, the time to cross the beam for the five trials as well as the total number of errors made (Brooks and Dunnett, 2009; Fleming et al., 2004). An "error" is considered a step that the mouse missed (i.e. placing its paw in imbalance) on the beam during crossing.

The pole test was performed to evaluate motor coordination (Drouin-Ouellet et al., 2012). Briefly, animals were placed head-up on top of a vertical 50-cm-high wooden pole with a diameter of 1 cm. Once placed on the pole, the time for the animal to orient itself downwards and the time to descend the entire pole length were calculated. A maximum of 30 seconds was allowed for the animal to both turn downward and climb down the pole. A 30-second score was attributed in case of a fall (Fleming et al., 2004). The animals received 2 days of training and were tested at a frequency of five trials per day.

For the hanging wire test (cable test), each animal was allowed to grab the wire by its forelimbs only. The time the mouse remained suspended was measured (60 seconds maximum). The test was performed 3 times, with a rest period of 30 seconds between each trial (Arsenault et al., 2011).

Mice were further tested using the open field to quantify general locomotion (Coulombe et al., 2016). The total distance travelled and speed of movements were recorded during a period of 60 minutes.

2.4 Tissue preparation

Animals were sacrificed at twelve months of age by intracardiac perfusion of phosphate buffer saline (PBS) containing inhibitors of proteases and phosphatases (Sigma-Aldrich) performed under deep anesthesia using ketamine and xylazine mixture (100 mg/kg and 10 mg/kg, respectively). Brains were collected and hemispheres separated. One side was snap frozen and stored at -80°C for cryostat sectioning into coronal sections of 20 µm in thickness, to dissect frozen samples from the striatum for Western blot and high-performance liquid chromatography (HPLC) analyses. Additional 12-µm thick sections from the same frozen hemisphere were prepared for *in situ* hybridization and autoradiography experiments. The other hemisphere was post-fixed in 4% paraformaldehyde (PFA) for 48 hours, transferred to 20% sucrose in 0.1 M PBS (pH 7.4) for cryoprotection and ultimately sliced at 25 µm using a freezing microtome (Leica Microsystems Inc., ON, Canada) (Bousquet et al., 2009; Bousquet et al., 2008).

2.5 Lipid extraction and gas chromatography

Brain fatty acid profiles were measured using approximately 10 mg of prefrontal cortex. Total lipids were extracted with 2:1 chloroform-methanol, saponified and transmethylated in a solution

of 14% methanolic boron trifluoride. Fatty acid profiles were determined by gas chromatography equipped with a flame ionization detector (model 6890; Agilent, Palo Alto, CA, USA) and a 50-m BPX-70 fused capillary column (SGE, Melbourne, Australia; 0.25 mm inner diameter, 0.25 μ m film thickness) (Plourde et al., 2009).

2.6 HPLC

Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were quantified by HPLC using electrochemical detection, as previously described (Bousquet et al., 2008). The anterior striatum between bregma levels 1.18 and 0.02 mm) (Paxinos and Franklin, 2008) was dissected and homogenized with 180 μ l perchloric acid (0.1 N). A volume of 60 μ l of supernatant was directly injected with a 717 plus autosampler automatic injector, driven by a 1525 binary pump, separated with an Atlantis dC18 column, and measured with a 2465 electrochemical detector equipped with a glassy carbon electrode (Waters Limited, Qc, Canada) linked to a chart recorder. Electrochemical potential was set to 5 nA. The mobile phase was composed of 8% methanol, 0.4 mM EDTA, 2 mM NaCl, 0.9 mM octanic sulfonic acid, 55 mM NaH₂PO₄ and pH was adjusted at 2.9. The mobile phase was delivered at a flow rate of 1.2 ml/minute with a mean pressure of 3369 psi. HPLC data were normalized to protein concentrations, as determined with a bicinchoninic acid protein assay kit using BSA as standard (Pierce, Rockford, IL) and according to the manufacturer's protocol.

2.7 Cortical protein levels and Western blot analyses

Homogenization of samples was performed in 8 volumes of lysis buffer (150 nM NaCl, 10 nM NaH₂PO₄, 1% v/v Triton X-100, 0.5% SDS, and 0.5% sodium deoxycholate) with a mix of protease (Roche, ON, Canada) and phosphatase inhibitors (1 mM tetrasodium pyrophosphate and 50 mM sodium fluoride). The samples were sonicated (3 x 10-s pulse) and centrifuged for

20 minutes at 100 000 g, 4°C. The supernatant was collected and frozen at -80°C. The quantification of protein concentration was performed using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Twelve µg of proteins were heated with Laemmli loading buffer for 5 minutes and separated by electrophoresis on an 8% SDS-polyacrylamide gel. Proteins were then transferred onto 0.45 µm Immobilon PVDF membranes (Millipore ON, Canada) and blocked with 5% skimmed milk, and 1% BSA in PBS - 0.05% Tween for 1 hour. The membranes were immunoblotted with the following primary antibodies: rabbit αSyn MJFR1 (Abcam; 1:1,000), mouse anti-CP13 (gift from Dr. Peter Davies, 1:5000), mouse anti-drebrin (Progen; 1:500), rabbit anti-NeuN (Abcam, ERP12763, 1:5000), mouse anti-postsynaptic density protein 95 (PSD95, Neuromab, CA, Neuromab; 1:5000), rabbit anti-septin (Novus biological; 1:20,000), mouse anti-synaptophysin (Millipore; 1:10 000), rabbit synaptosomal-associate protein 25 (SNAP25 Covance; 1:20,000), rabbit anti-Tau C-Terminal (Tau C, Dako; 1:20,000), rabbit anti-tyrosine hydroxylase (TH, Pel-Freez, AR, USA, 1:5000), mouse anti-VGLUT1 (Neuromab, 1:4000) and a loading control mouse anti-actin (ABM; 1:10,000). After washing the membranes in PBS-Tween, they were incubated with the appropriate HRP-coupled anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch, PA, USA) followed by detection with chemiluminescence reagent (Luminata, Millipore) which was measured with myECL Imager (Thermo Fisher Scientific Inc.). Immunoblot band intensity was quantified with Carestream Molecular Imaging Software (Molecular Imaging Software version 4.0.5f7, Carestream Health, NY, USA).

2.8 Autoradiography

Striatal dopamine transporter (DAT) specific binding (Bregma level -0.46 mm) was evaluated using ¹²⁵I-RTI-121 [3β-(4-¹²⁵I-iodophenyl) tropane-2β-carboxylic acid isopropyl ester] (NEN-DuPont, MA, USA; 2200 Ci/mmol) binding according to previously published procedures

(Bousquet et al., 2008; Calon et al., 2001). Sections were preincubated at room temperature for 30 minutes in potassium phosphate buffered saline (KPBS: 10.1 mM NaHPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, and 2 mM KCl pH 7.4) followed by a 60-minute incubation at room temperature with 20 pM ¹²⁵I-RTI-121. Mazindol (10 nM) was used to evaluate non-specific binding (Novartis, Basel, Switzerland). After two 20-minute washes in PBS, sections were briefly (10 seconds) rinsed in distilled water (all at 4°C). Finally, the slide-mounted tissue sections were dried overnight at room temperature and exposed on Kodak Biomax film (Sigma-Aldrich) for 24 hours.

2.9 Immunohistochemical evaluation of the nigro-striatal dopaminergic system

PFA-fixed sections from the substantia nigra pars compacta (SNpc) and the striatum described above were washed in 0.1 M PBS and subsequently placed in 3% hydrogen peroxide for 30 minutes at room temperature. Sections were washed in PBS and placed in a blocking solution (PBS, 0.1% Triton X-100 (Sigma-Aldrich) containing 5% normal goat serum (NGS; Wisent, Qc, Canada) for 30 minutes. The sections were then incubated overnight at 4°C with a rabbit primary antibody anti-TH (PEL-Freez, 1/5000 for SNpc and 1/500 for striatum) in blocking solution. Sections were washed three times in PBS, incubated with a secondary biotinylated goat anti-rabbit IgG (Jackson Immunoresearch) followed with an incubation with an avidin-biotin peroxidase complex (ABC; Vector Laboratories, CA, USA) for 1 hour at room temperature. The antibody binding was detected by placing the sections in a solution of 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) and 0.1% of 30% hydrogen peroxide in PBS. The reaction was stopped by extensive washes in PBS. Following the DAB staining, sections were counterstained with cresyl violet (sigma-Aldrich), dehydrated and coverslipped.

2.10 Quantification of nigral TH-immunoreactive neurons

The number of nigral TH+ neurons was determined by stereological counts under bright-field illumination, as previously described (Bousquet et al., 2008). Seven sections immunostained for TH and selected through the SNpc (AP: levels of -2.70 mm to -3.80 mm) were analyzed using the Stereo investigator software (MicroBrightfield) integrated to an E800 Nikon microscope (Nikon Canada Inc., ON, Canada) (Gibrat et al., 2009). Two independent investigators, blinded to experimental conditions, performed unbiased cell counts. Analyses of immunoreactive profiles were restricted to the SNpc and thus excluded the ventral tegmental area.

2.11 Statistical analyses

Results for each group are presented as the mean \pm SEM. For Western blot data, values that were within one standard deviation from the background values were excluded. In cases of comparable group variances, as established by a Bartlett's test, statistical analyses were performed using one-way analysis of variance (ANOVA) followed by post-hoc (Tukey's) tests for comparisons between groups. To compare diet and genotype as independent categorical variables, we performed two-way ANOVA. For comparisons between two groups, a Student's t-test was used. Mann-Whitney test was selected in cases where variances were not equivalent. These were identified using the ROUT method, based on the analyses of non-linear regression and evaluation of the residuals of the robust fit. All statistical analyses were performed using the JMP (version 11; SAS Institute Inc., Cary, IL, USA) and Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) softwares. A *p* value of 0.05 was considered statistically significant.

3. RESULTS

Accumulation of cerebral DHA levels following exposure to a high n-3 PUFA diet

High dietary intake of n-3 PUFAs over a period of 10 months in mice (from two to twelve months of age) selectively increased frontal cortex levels of DHA (**Table 2**), confirming treatment

efficacy. Dietary intake of DHA decreased levels of total n-6 PUFA, arachidonic acid and adrenic acid. The n-3 PUFA:n-6 PUFA ratio also increased with the treatment enriched with DHA (**Table 2**). The opposite occurred with an increase of total n-6 PUFA and a decrease of DHA observed in animals fed the diet that did not contain DHA, along with a lower n-3 PUFA:n-6 PUFA ratio. There was no effect of genotypes on fatty acid profiles (**Table 2**).

Impact of a DHA enriched diet on mice longevity

We first assessed whether n-3 PUFA-enriched diet intake improved motor behavior of Thy1- α Syn mice. Motor performance was evaluated at 4, 8 and 12 months (**Figure 1**). Thy1- α Syn mice made more errors when crossing the beam than the non-transgenic mice (**Figure 1B**). Thy1- α Syn mice took longer to perform the pole test than their control counterparts, particularly with advancing age (**Figure 1C**). DHA treatment aggravated this phenotype in Thy1- α Syn mice at each time point, particularly at 4 months of age (**Figure 1C**). Muscle tone was not modified by the diets (hanging wire test). At 4 and 8 months, non-transgenic mice remained suspended to the wire for a longer period of time than Thy1- α Syn mice (**Figure 1D**). The transgene significantly decreased the motor fluency of the mice at the three ages evaluated when the spontaneous activity test was performed (**Figure 1E**). In general, Thy1- α Syn mice traveled greater distances than non-transgenic mice (**Figure 1F**). Thy1- α Syn mice on the control diet evolved towards reduced hyperactivity at 12 months, consistent with previous observations (Lam et al., 2011). The high-DHA diet significantly prevented this decline, although a similar non-significant trend was also seen with animals on the low n-3 PUFA diet (**Figure 1F**). Remarkably, DHA treatment prolonged the survival of Thy1- α Syn mice (**Figure 1G**). More than 33% of the Thy1- α Syn mice not supplemented with DHA died before reaching 6 months of age, compared to 6% for DHA-fed Thy1- α Syn mice. The survival medians for each group were 12, 9, 6 and 12 months for non-transgenic littermates, Thy1- α Syn on control diet, low n-3 PUFA diet (no DHA)

or high-DHA diets, respectively (**Figure 1G**), indicating that the majority of DHA-treated Thy1- α Syn mice lived until the fixed sacrifice time. In sum, despite the absence of consistent behavioral effects, DHA intake clearly improved the survival of Thy1- α Syn mice.

Confirmation of human α Syn in the Thy1- α Syn brain

Human α Syn was detected only in brain homogenates derived from Thy1- α Syn and not from control C57BL/6 mice, using a rabbit antibody raised against human α Syn (MJFR1) (**Figure 2**). Both soluble (**Figure 2A**) and insoluble (**Figure 2B**) protein fractions were quantified by Western blot and confirmed the expression of human α Syn in the cortex of Thy1- α Syn mice. The different forms of oligomers were identified depending on the band heights on the Western blots (**Figure 2**). No significant effect of dietary treatment on the levels of human α Syn was observed, aside from higher 42-kDa insoluble oligomer in Thy1- α Syn mice under the DHA deprived diet compared to controls or to those supplemented with DHA (**Figure 2B**). This change in the α Syn-positive band migrating at 42-kDa should be interpreted with caution, as its levels were very low.

Impact of dietary treatments on the nigro-striatal system

Three components of the nigro-striatal dopaminergic system were studied: striatal DAT terminals, levels of dopamine and metabolites as well as TH-immunoreactivity in the SNpc. No significant difference was observed between dietary groups in DAT (**Figure 3A,B**) nor TH analyses in Thy1- α Syn mice (**Figure 3C,D**). Interestingly, lower SNpc TH signal and DAT levels were detected in Thy1- α Syn mice compared to the C57BL/6 mice (both under the control diet) when a planned comparison t-test was performed. A two-way ANOVA also revealed a significant effect of the Thy1- α Syn genotype on lowering nigral TH immunostaining and DAT levels, but no effect of the dietary treatment. Western blot analyses did not reveal a difference in striatal TH

levels between genotypes (data not shown). No significant difference in dopamine concentrations was detected by HPLC analysis between Thy1- α Syn and non-transgenic C57BL/6 littermates under the control diet. However, an increase of striatal dopamine levels was observed in Thy1- α Syn mice fed with enriched DHA diet comparatively to other groups (**Figure 3E**). Dopamine metabolites did not show any significant difference between groups (**Figure 3F and G**), and only lower DOPAC/DA ratio reached statistical significance in DHA-treated Thy1- α Syn mice (**Figure 3H-I**)

Changes in synaptic proteins with mice genotypes

Western blot analysis showed altered synaptic protein levels between genotypes (**Figure 4**). A decrease of synaptophysin, PSD95, VChAT and NeuN was observed in the cerebral cortex for all Thy1- α Syn groups regardless of the dietary intake (**Figure 4A,D,G,H**). Unexpectedly, enriched DHA diet also brought a decrease of SNAP25 in transgenic mice (**Figure 4B**). However, drebrin, NeuN and septin concentrations remained unchanged (**Figure 4C,E, F**).

4. DISCUSSION

The goal of this study was to extend our prior data demonstrating the beneficial effects of n-3 PUFA enriched diets in toxin-based animal models of PD to a mouse model of brain synucleinopathy. We therefore evaluated the effects of a nutraceutical intervention with DHA in the Thy1- α Syn mouse model of PD. Our results demonstrate 1) a significant impairments in motor behavior and lower survival rates in a large cohort of Thy1- α Syn mice, 2) that brain α Syn accumulation in Thy1- α Syn mice is associated with a robust decrease in the key synaptic proteins synaptophysin and PSD95, further accompanied by partial deficits in dopaminergic-

related parameters, 3) that dietary DHA has a significant beneficial effect on the longevity of mice associated with higher dopamine levels, but with limited effects on behavioral impairment and no concomitant significant effect on brain α Syn content or synaptic protein levels.

Consistent with previous studies in rodents, the relative concentrations of fatty acids in the brain were profoundly altered by dietary intake of n-3 PUFA (Bourre, 1984; Bousquet et al., 2008; Coulombe et al., 2016; Lalancette-Hébert et al., 2011; Salem, 1989). More specifically, the increase of DHA measured in the prefrontal cortex confirmed the efficacy of the dietary treatment to impact brain fatty acid content. However, DHA accumulation in cerebral tissues was not clearly translated into detectable effects on motor impairments. On the one hand, DHA-treated Thy1- α Syn mice performed even more poorly than those on the control diet on the hanging wire or the pole tests. On the other hand, the dietary DHA treatment prevented the decline in activity observed at 12 months in Thy1- α Syn mice on the control diet. This latter observation can be interpreted as a slower progression towards parkinsonism (Lam et al., 2011). This should be taken with caution, however, as a similar non-significant trend was observed in animals on the low n-3 PUFA diet. The absence of clear motor improvement following DHA intake and the resulting dopamine increase is consistent with previous observations that the motor impairments observed in the Thy1- α Syn mice are not directly dependent on dopaminergic defects (Fleming, 2006; Richter et al., 2014).

A particularly striking observation was that the DHA treatment enhanced survival of the Thy1- α Syn mouse model of synucleinopathy. The 50% mortality rate observed in Thy1- α Syn mice fed the control or DHA-deprived diets was more important than in previous reports, where rising mortality was observed only after 14 months of age (Chesselet et al., 2012; Lam et al., 2011). It should be noted that we used Thy1- α Syn mice on a fully backcrossed C57Bl/6 background,

while most publications use the hybrid C57BL/6-DBA/2 background (Chesselet et al., 2012; Lam et al., 2011; Magen et al., 2015), which may have contributed to the higher and earlier mortality rate observed here. Although we did not determine the exact causes of mortality, the protective effect of the DHA diet may relate to its multiple beneficial effects on different organs, aside from the brain, during aging (Trepanier et al., 2016).

As expected, we further confirmed the expression of human α Syn in the cortex of Thy1- α Syn mice in the form of monomers, as well as various soluble and insoluble oligomers of 42, 110 and 260 kDa in size. Dietary treatment had only a minimal effect on cortical levels of human α Syn monomers and oligomers with the exception of the 42-kDa insoluble oligomer, which was found in higher concentrations in mice fed the low n-3 PUFA diet. It has been proposed that PUFA integration in membrane phospholipids alters α Syn associations with cell membrane as well as in α Syn oligomerization and aggregation (Sharon et al., 2003b). Indeed, experiments on dopaminergic or neuronal cell lines indicate that PUFA regulate oligomerization of monomers into higher weight soluble complexes, which could precede Lewy body-like inclusion formation (Assayag et al., 2007; Sharon et al., 2003a). More recent findings suggest that dietary alterations in brain DHA levels affect α Syn cytopathology in transgenic mice for the PD-causing A53T mutation in human α Syn (Yakunin et al., 2012). In addition, there is evidence that these associations are involved in the potential physiological function of α Syn, activating mechanisms of membrane trafficking and specifically, synaptic vesicle recycling (Emanuele and Chieregatti, 2015). Nevertheless, the pathogenic role of this specific 42-kDa oligomer present in weak concentration in the brain of these mice remains unclear. A similar α Syn band migrating at a 42-kDa range has been previously observed in the cerebrospinal fluid collected from both PD patients and controls (Jakowec, 1998). Although neurotoxicity has been attributed mostly to soluble α Syn oligomers, the relative importance of each oligomer, soluble or insoluble, in how

they may contribute to the progression to parkinsonism remains unclear (Barrett and Greenamyre, 2015; Bartels et al., 2011). Most evidence suggests that the equilibrium between monomers and tetramers (58 kDa) is particularly important to the initiation of α Syn misfolding and human synucleinopathies (Barrett and Greenamyre, 2015; Bartels et al., 2011). As such, the change in 42-kDa oligomer observed in our study may represent a key step affected by DHA in the formation of oligomers of larger size or in the process of fibril formation.

Consistent with previous findings, our results evidence only weak decreases in dopaminergic markers in the Thy1- α Syn transgenic model, regardless of the dietary treatment. A significant decrease of dopaminergic neurons in the SNpc was nonetheless observed at 12 months of age in Thy1- α Syn compared to non-transgenic C57BL6, particularly when statistical power was increased by pooling all transgenic mice, and ignoring dietary treatment. It is possible that the aggregation of human α Syn could potentially need to reach a certain threshold before inducing degeneration of the dopaminergic system. Our results are thus consistent with the hypothesis that motor dysfunction in the Thy1- α Syn model is not related to dopamine deficits, but to other causes such as alterations in corticostriatal synaptic function (Watson et al., 2009), changes in gut microbiota and/or central microglia activation (Sampson et al., 2016). Notably, however, we observed that DHA treatment stimulates secretion of dopamine in the striatum. This is in agreement with reported indication of enhanced dopaminergic drive in animals fed with n-3 PUFA (Bousquet et al., 2011a; Chalon, 2006; Coulombe et al., 2016; Delattre et al., 2010; Mori et al., 2017). The fact that this was not translated into improved behavior remains consistent with previous reports that early behavioral deficits in these mice are worsened by either dopaminergic agonists (Fleming, 2006) or increased expression of genes associated with the dopaminergic system (Richter et al., 2014).

The Thy1- α Syn model also showed unexpected reductions in two synaptic proteins in the cerebral cortex, namely synaptophysin and PSD95. This observation is consistent with results of studies in primary hippocampal neurons showing that exposure to human α Syn induces presynaptic toxicity with a decrease in key synaptic proteins, including synaptophysin (Volpicelli-Daley et al., 2011). This is further in line with neuropathological observations indicating that an important proportion of α Syn aggregates are localized in axons in human synucleinopathies, leading to the suggestion that α Syn induces synaptic dysfunction before neurodegeneration (Lam et al., 2011; Schulz-Schaeffer, 2010; Wu et al., 2010).

Our results reinforce the conclusion that despite a widespread accumulation and aggregation of α Syn in the brain, the Thy1- α Syn transgenic model does not present a robust dopaminergic degeneration (Chesselet et al., 2012), but rather a loss of synaptic proteins which may be relevant to corticostriatal circuit dysfunction observed in early PD (Lam et al., 2011; Rodriguez-Oroz et al., 2009; Wu et al., 2010). In the present study, DHA dietary treatment did not decrease the amount of human α Syn present in the brain of Thy1- α Syn mice, neither did it protect the integrity of synaptic proteins. Since no frank dopaminergic loss was observed in this model, it is logical that DHA-induced increase in striatal dopamine did not translate into obvious motor impairments. Nevertheless, the DHA enriched diet lengthened the survival of Thy1- α Syn mice, consistent with a protective action downstream of α -synucleinopathy. Additional investigations will be necessary to validate the potential of a DHA-based nutraceutical intervention in PD patients but the present data does not suggest that an n-3 PUFA mechanism of action includes improving cerebral α -synucleinopathy.

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FIGURE LEGENDS

Figure 1. Impact of n-3 PUFA on motor behavior and longevity. (A) Time line of experimentation. The different behavioral tests revealed a decrease of motor abilities of the Thy1- α Syn transgenic mice with age; deficits that were not restored with high DHA intake (B-F). However, diets had a significant impact on the percentage of mice that survived up to 12 months of age (G). More than 50% of transgenic mice died when fed with low n-3 PUFA diet compared to C57BL/6 control mice. In contrast, animals fed a high-DHA had an attrition of only 25% at 12 months of age (G). Statistical analyses were performed using One-Way ANOVA.

* P<0.05, ** P<0.01, **** P<0.0001 for C57BL/6 control mice compared to Thy1- α Syn mice on control diet;

P<0.01, ### P<0.001, #### P<0.0001 for Thy1- α Syn mice on Low n-3 PUFA diet (No DHA) compared to C57BL/6 control mice;

\$ P<0.05 \$\$ P<0.01, \$\$\$ P<0.001, \$\$\$\$ P<0.0001 for Thy1- α Syn mice on DHA diet compared to C57BL/6 control mice;

£ P<0.05 ££ P<0.01 £££ P<0.001 for Thy1- α Syn mice on DHA diet compared to Thy1- α Syn mice on control diet;

@ P<0.05 @@ P<0.01 for Thy1- α Syn mice on DHA diet compared to Thy1- α Syn mice on Low n-3 PUFA diet (No DHA). Values are expressed as means \pm S.E.M.

Figure 2. Impact of n-3 PUFA on human α Syn levels in cerebral tissue. Cortical soluble and insoluble fractions of α Syn oligomers were quantified by Western blot. As expected, control mice depicted negligible immunoreactivity for the antibody targeting human α Syn (A-B). Additionally, diets had a very small impact on α Syn levels, which was limited to an increase of the 42-kDa insoluble oligomer in animals fed a low n-3 PUFA diet (no DHA) compared to control (C57 Ctrl) or DHA-treated animals (DHA) Thy1- α Syn mice. (B). Statistical analyses were performed using One-Way ANOVA. * P<0.05, ** P<0.01, *** P<0.001 **** P<0.0001 compared to background levels; # P<0.05 compared to DHA-fed Thy1- α Syn mice. @ P<0.05 for Thy1- α Syn mice on DHA diet compared to Thy1- α Syn mice on a low n-3 PUFA diet (No DHA). n.d. : non detectable Values are expressed as means \pm S.E.M. Values that were within one standard deviation from the background values were excluded, which accounts for the different number of animals in each group for each analysis. While the example in B is from a single exposure of the membrane, relative optical densities were analyzed from different exposure to compare groups, thus do not reflect the relative amount of monomers versus each oligomer within samples.

Figure 3. Impact of n-3 PUFA on the nigro-striatal system. Thy1- α Syn mice exposed to DHA had higher levels of striatal dopamine compared to non-transgenic (C57BL/6, Ctrl) or to Thy1- α Syn mice fed the n-3 PUFA-depleted diet (no DHA) (A) No significant changes in the ratio between metabolites and dopamine were detected (B). Genetic expression of human α Syn led to a decrease in 125 I-RTI-121 specific binding to DAT (A) and in the number of TH-immunopositive dopaminergic nigral cells (C) using a 2-way ANOVA. When statistical analyses were performed using a One-Way ANOVA, the decrease in both dopaminergic markers was significant only in transgenic mice fed with the control diet, compared to C57BL/6 control mice. Representative images of 125 I-RTI-121 specific binding to DAT and TH immunostaining in the mouse substantia nigra (B-D). * P<0.05, ** P<0.01, compared to non-transgenic C57BL/6 control mice using a 1-way ANOVA (Tukey's post-hoc test), # P<0.05 compared to non-transgenic C57BL/6 control mice analyses using a 2-way ANOVA, †† P<0.01 versus Low n-PUFA-fed

Thy1- α Syn mice (no DHA), using a 1-way ANOVA (Tukey's post-hoc test). Values are expressed as means \pm S.E.M. Abbreviations: DOPAC: 3,4-dihydroxyphenylacetic acid, HVA: homovanillic acid, DA: dopamine, DAT: dopamine transporter, TH: tyrosine hydroxylase.

Figure 4. Impact of n-3 PUFA on synaptic and neuronal markers. Lower concentrations of synaptic proteins synaptophysin, VChAT, PSD95 and NeuN were observed in the cerebral cortex of Thy1- α Syn mice compared to non-transgenic C57BL/6 mice, independently of the dietary intake of a control (Ctrl), low n-3 PUFA (No DHA) or high-DHA (DHA) diets. (**A, D, G, H**). SNAP25 decreased in the Thy1- α Syn mice fed with DHA enriched diet compared to C57BL/6 control groups (**B**). Drebrin and septin-3 remained unchanged (**C, E**). Actin was used as loading control and remained unchanged between each group (**F**). Western blot bands were cropped from the same membrane. Statistical analyses were performed using One-Way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to C57BL/6 control mice. & $P < 0.05$, && $P < 0.01$, &&& $P < 0.0001$ compared to C57/BL6 control mice using a two-way ANOVA. Values are expressed as means \pm S.E.M.

Table 1 - Description of dietary

	Control	Low n-3 PUFA	High DHA
	Medium LNA	Low LNA	Low LNA
	No DHA	No DHA	High DHA
Protein (% w/w)	20.3	20.3	20.3
Carbohydrate (% w/w)	66.0	66.0	66.0
Fat (% w/w)	5.0	5.0	5.0
Calorie per diet weight kcal/g	3.9	3.9	3.9
Ingredients (g/kg)			
Casein	200	200	200
DL-Methionine	3	3	3
Corn Starch	150	150	150
Sucrose	500	500	500
Cellulose, BW200	50	50	50
Corn Oil	30	25	10
Safflower Oil	0	25	19
DHA powder KSF58	0	0	50
Soybean Oil	10	0	0
Canola oil	10	0	0
Fatty acids (g/kg)¹			
Total n-3 PUFA	1.43	0.31	7.80
LNA	1.42	0.28	0.14
EPA	0	0	0.26
DHA	0	0	7.20
Total n-6 PUFA	21.20	30.29	18.42
Linoleic acid	21.10	30.14	16.65
Arachidonic acid	0	0	0.06
n-6:n-3 PUFA ratio	14.82	97.70	2.36

Abbreviations and notes:

- n-3 and n-6 = n-3 and n-6 polyunsaturated fatty acids (PUFA)
- DHA: docosahexaenoic acid (22:6n-3) from DHA microencapsulated powder (Martek/DSM)
- EPA: eicosapentaenoic acid (20:5n-3)
- LNA: alpha-linolenic acid (18:2 n-3) (from soybean oil)

¹ Determined by gas chromatography after lipid extraction of each diet

Table 2 – Prefrontal cortex fatty acid composition determined using gas chromatography

	C57BL/6 Control diet	Thy1-αSyn Control diet (No DHA)	Thy1-αSyn Low n-3 PUFA (No DHA)	Thy1-αSyn High DHA	
% total fatty acids					
N	8	8	8	8	
C16:0	25.54 \pm 0.19	25.37 \pm 0.42	25.40 \pm 0.15	25.14 \pm 0.14	
C18:0	21.69 \pm 0.56	20.56 \pm 0.80	20.72 \pm 0.71	20.37 \pm 0.85	
Total SFA	47.22 \pm 0.56	45.94 \pm 0.92	46.12 \pm 0.69	45.51 \pm 0.81	
C18:1 n-9	16.42 \pm 0.23	16.40 \pm 0.43	16.06\pm0.48 **	16.50 \pm 0.37	
Total MUFA	17.70 \pm 0.23	17.88 \pm 0.44	20.16 \pm 0.56	19.37 \pm 0.36	
C18:2 n-6 LA	0.74 \pm 0.08	0.73 \pm 0.03	0.79 \pm 0.04	0.60 \pm 0.03	
C20:4 n-6 ARA	11.74 \pm 0.27	12.22 \pm 0.33	12.37\pm0.48	10.05\pm0.23 ††† ***	
C22:4 n-6 DTA	3.05 \pm 0.06	3.12 \pm 0.11	3.43\pm0.22	1.89\pm0.052 †††† ****	
Total n-6 PUFA	16.49 \pm 0.99	16.08 \pm 0.35	16.59\pm0.66	12.54\pm0.27 ††† **	
C22:6 n-3 DHA	19.54 \pm 0.40	20.10 \pm 0.61	16.85\pm0.97 * *	22.58\pm0.41 †††† *	
Total n-3 PUFA	19.54 \pm 0.40	20.10 \pm 0.61	16.85\pm0.97 **	22.58\pm0.41 †††† *	
n-3:n-6 ratio	1.21 \pm 0.06	1.25 \pm 0.04	1.04\pm0.11	1.80\pm0.04 †††† ****	
Total PUFA	36.03 \pm 1.09	36.18 \pm 0.84	33.44 \pm 0.91	35.12 \pm 0.56	

††† P < 0.001, †††† P < 0.0001 versus low n-3 PUFA diet (same genotype)

* P < 0.05, **P < 0.01, *** < 0.001, **** < 0.0001 versus non-transgenic mice (Ctrl diet)

C16:0; palmitic acid, C18:0; stearic acid, SFA; saturated fatty acid, C18:1 n-9; Oleic acid, MUFA; monounsaturated fatty acid, C18:2 n-6 (LA); Linoleic acid, C20:4 n-6 (ARA); Arachidonic acid, C22:4 n-6 (DTA); Docosatetraenoic acid, n-6 PUFA; omega-6 polyunsaturated fatty acids, C22:6 n-3 (DHA); Docosahexaenoic acid, n-3 PUFA; omega-3 polyunsaturated fatty acids, n-3; omega-3 polyunsaturated fatty acids, n-6; omega-6 polyunsaturated fatty acids.

FIGURE 1

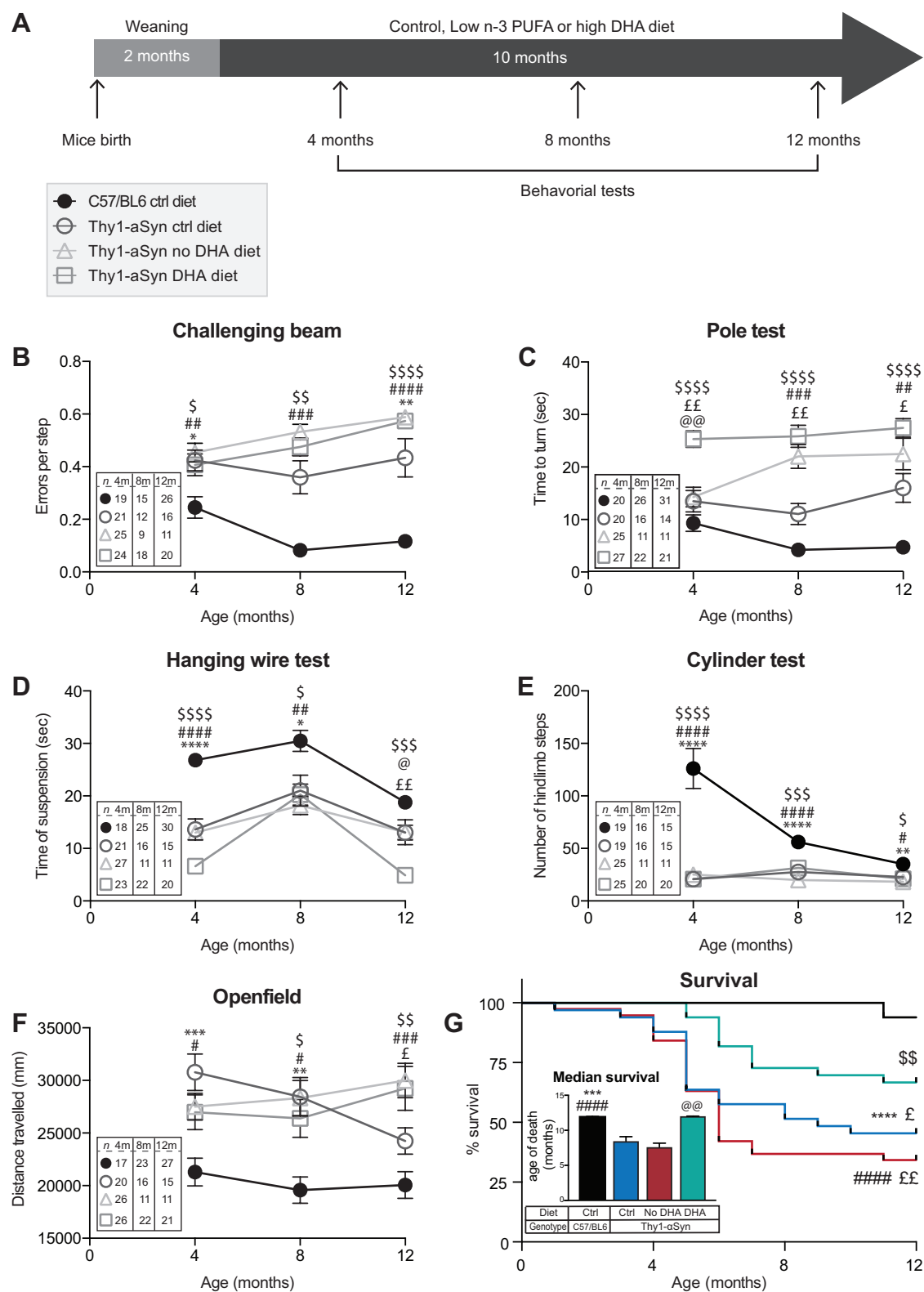
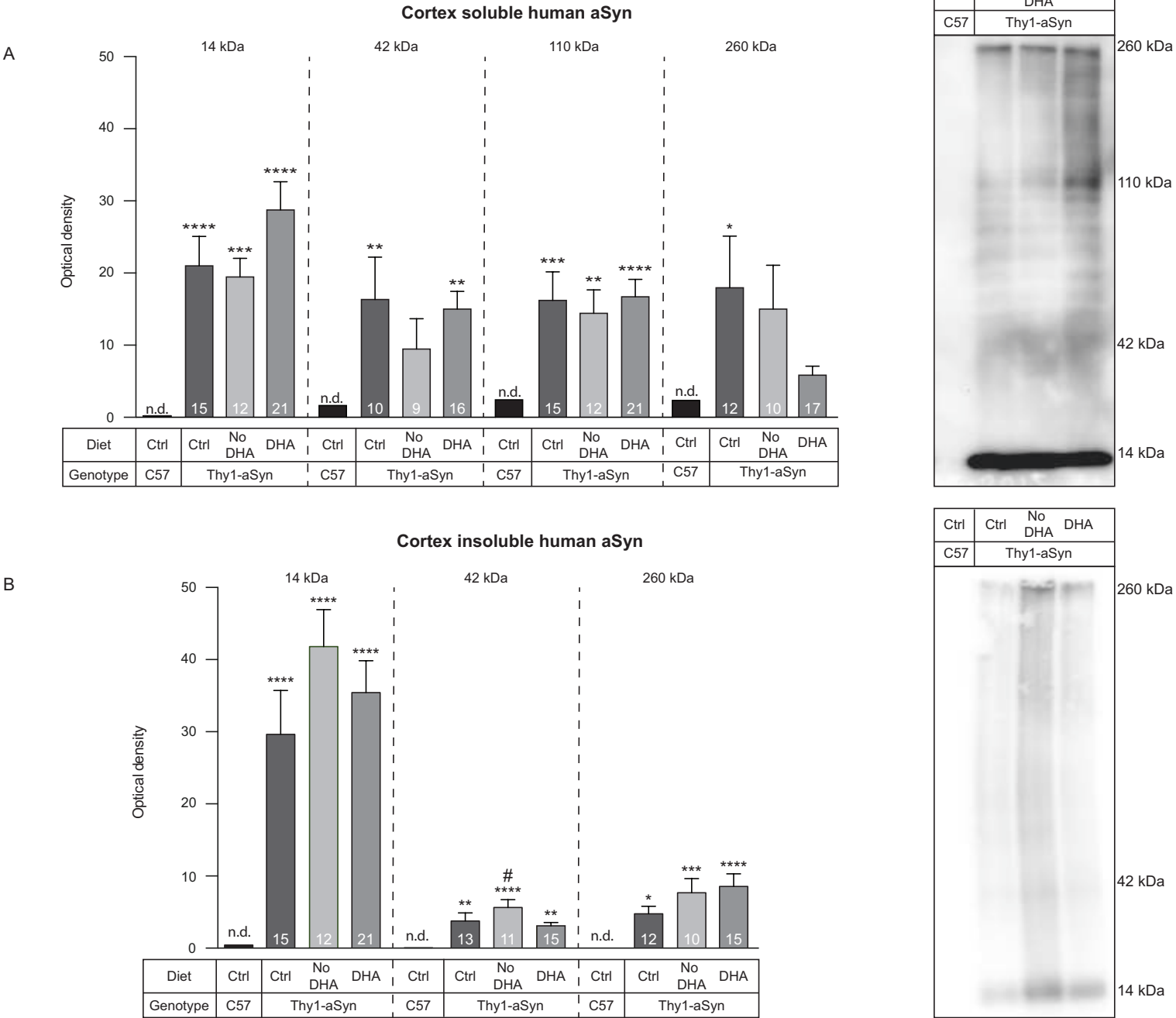
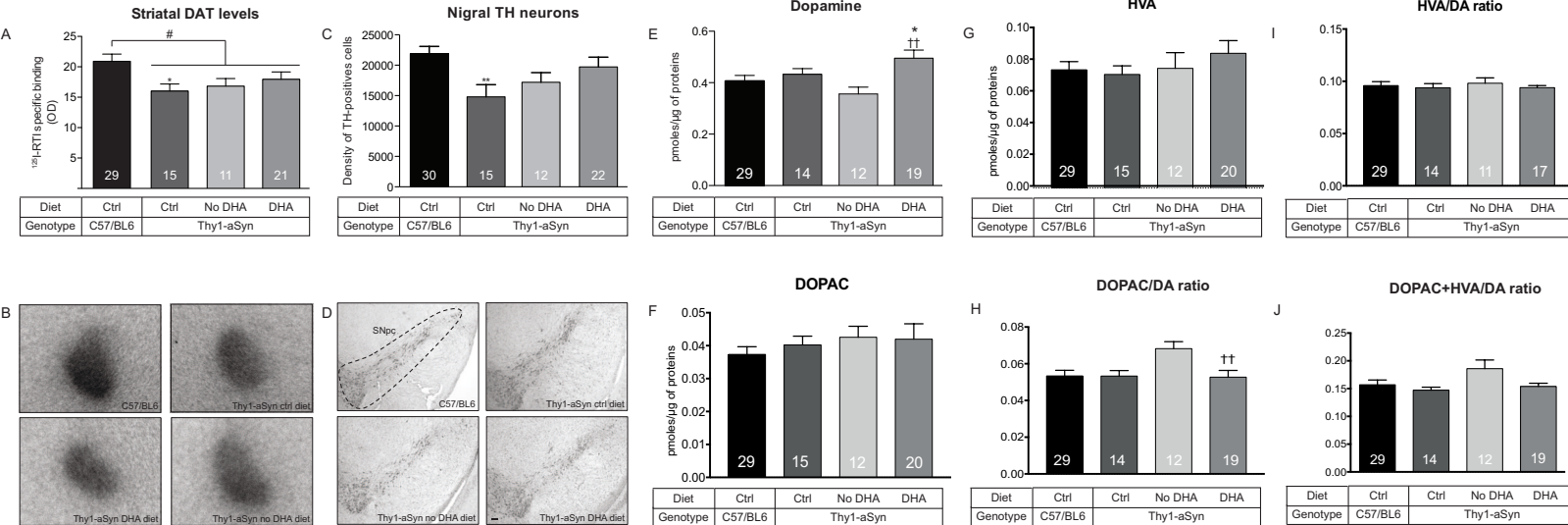


FIGURE 2



Figure_3
Click here to download Figure: FIGURE 3_RTI_TH_HPLC_final01.eps

FIGURE 3



* P < 0.05 versus NonTg Ctrl
†† P < 0.01 versus Low n- PUFA

FIGURE 4

